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Cardio-respiratory alterations following acute normovolemic hemodilution in a pediatric and an adult porcine model: A prospective interventional study

--Manuscript Draft--

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Abstract:	Background: Acute normovolemic hemodilution (ANH) is considered as a blood sparing intervention during the perioperative management. We aimed at comparing the cardiopulmonary consequences of ANH between adult pigs and weaned piglets to establish the effects of lowering hematocrit (Hct) in these age groups, and thereby testing the hypothesis that difference in the age-related physiological behavior will be reflected in the cardiorespiratory changes following ANH. Methods: ANH was achieved in anesthetised, mechanically ventilated adult minipigs and 5-weeks old weaned piglets by stepwise blood withdrawal (10 ml/kg) with crystalloids replacement. Cardiorespiratory assessments consisted of measuring airway resistance (Raw), respiratory tissue elastance (H), effective lung volume (ELV), extravascular lung water (EVLW), mean arterial pressure (MAP), pulmonary blood flow (PBF) and cardiac output (CO). Respiratory and hemodynamic measurements were made at control conditions and following each ANH condition obtained with 5 to 7 steps. Results: ANH induced immediate and progressive increases in Raw and H in both groups with more pronounced worsening in adults despite the similar decreases in Hct. The increases in EVLW were significantly greater in the adult population with the differences in mean (DM) of 25.1% (95% CI 5.3%-44.9%). Progressive ANH led to significant decreases in the DM of PBF (45.3% (95% CI 19.8%-70.8%) and MAP (36.3% (95% CI 18.7%-53.9%) only in adults, whereas CO increased significantly only in the piglets (DM: 51.6 (95% CI 14.2%-89.0%)). Conclusions: While ANH led to mild detrimental cardiorespiratory changes in weaning piglets, gradual developments of bronchoconstriction, lung tissue extravasation and

	stiffening, and deteriorations in systemic and pulmonary hemodynamics were observed in adults. ANH may exert age-dependent cardiorespiratory effect.
Response to Reviewers:	Reply to the Statistical Editor: “I appreciate the opportunity to re-review your manuscript. The manuscript is now much improved. Please find below a single area in which the manuscript can be further clarified or improved from the statistical methods and/or reporting perspective.” Reply 1: We thank again the Statistical Editor for the comments contributing greatly to the clarification of the statistical issues in the manuscript. Please find our point-by-point replies for the few remaining points below. “1. P8L12-30. Thank you for including a sample size justification. As previously requested, please include the section at the end of statistical methods, after statistical methods are explained, not at the beginning.” Reply 2: We apologize for the miscomprehension; the sample size justification has been moved to the end of the statistical methods in the revised manuscript. “Authors say “considering 25% difference between the groups is clinically significant”, but is not clear whether this is an absolute or relative percent difference. Please visit Pacific in delta you are using.” Reply 3: We specify in the revised manuscript that this difference was meant as absolute difference between the percentage changes. “Instead of “P level of 0.05” say that you set alpha to be 0.05. At this place (not earlier) also tell the reader about your adjustment for multiple comparisons and how that impacted the sample size calculation.” Reply 4: All these corrections have been made in the revised manuscript. Reply to Reviewer 1: “Reviewer 1: The investigators have made substantial improvements in this manuscript based on the editors and reviewers’ suggestions. The statistical analysis is especially robust now. They have addressed most of my comments and concerns. I would like the limitations to be stated more clearly in the discussion and have the following suggestions: Discussion: Suggest revising the heading “Methodological considerations” to “Methodological considerations and limitations”. This section should be put at the end of the discussion.” Reply 1: We thank again the Reviewer for the thoughtful comments contributing greatly to the improvement of our manuscript. In agreement with this suggestion, this chapter was moved to the end of the discussion and the title has been expanded with the limitations (page 14, paragraphs 2-3). ““Re the paragraph; “It is of note that the blood volume expressed relative to body weight is about 30% greater in piglets than for adults…….” I still think this statement/paragraph is confusing and needs a little more clarification. Please expand on the “the blood volume expressed relative to body weight is about 30% greater in piglets than for adults” What exactly is the blood volume per kg for each age group? Please explain what “the level of targeted Hct” is exactly and how it was “comparable between groups”.” Reply 2: We referred in the manuscript to the studies that determined the blood volume in piglets (90 +/- 6.1 ml/kg) and in pigs (67.3 +/- 3.67 ml/kg). We believe that this difference may explain the initial steeper drop in hematocrit observed in the adult pigs. However, when mentioning the level of targeted hematocrit, we meant the level obtained following hemodilution. Our goal was to reach no statistically significant differences in the hematocrit values between the protocol groups after each step of hemodilution, as it is demonstrated on the top left panel of Fig. 3. “Still I am not sure you can prove that the degree of hemodilution is no different between groups. From my line of reasoning; the adults may have had a larger proportion of blood removed and therefore a larger proportion of crystalloid replaced relative to their body weight compared to the piglets. Hemodilution may have been

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CARDIO-RESPIRATORY ALTERATIONS FOLLOWING ACUTE NORMOVOLEMIC HEMODILUTION IN A PEDIATRIC AND AN ADULT PORCINE MODEL:
A prospective interventional study

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Conflict of interest: A Servo-i ventilator with additional software was provided by Maquet Critical Care (Solna Sweden). No additional conflict of interest.

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Abbreviated title: Age-dependency of hemodilution

Authors' contributions:
GA: data collection, data analyses, article drafting
CS: protocol design, article drafting
MD: protocol design, article drafting
MW: data collection, data analyses, article drafting
FP: statistical analyses, interpretation of the results, article drafting
WH: protocol design, interpretation of the results, article drafting

ABSTRACT

Background: Acute normovolemic hemodilution (ANH) is considered as a blood sparing intervention during the perioperative management. We aimed at comparing the cardiopulmonary consequences of ANH between adult pigs and weaned piglets to establish the effects of lowering hematocrit (Hct) in these age groups, and thereby testing the hypothesis that difference in the age-related physiological behavior will be reflected in the cardiorespiratory changes following ANH.

Methods: ANH was achieved in anesthetised, mechanically ventilated adult minipigs and 5-weeks old weaned piglets by stepwise blood withdrawal (10 ml/kg) with crystalloids replacement. Cardiorespiratory assessments consisted of measuring airway resistance (Raw), respiratory tissue elastance (H), effective lung volume (ELV), extravascular lung water (EVLW), mean arterial pressure (MAP), pulmonary blood flow (PBF) and cardiac output (CO). Respiratory and hemodynamic measurements were made at control conditions and following each ANH condition obtained with 5 to 7 steps.

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Conclusions: While ANH led to mild detrimental cardiorespiratory changes in weaning piglets, gradual developments of bronchoconstriction, lung tissue extravasation and stiffening, and deteriorations in systemic and pulmonary hemodynamics were observed in adults. ANH may exert age-dependent cardiorespiratory effect.

INTRODUCTION

Anesthesiologists are frequently challenged by the perioperative indication for blood transfusion. Despite heterogeneous results highlighted in a recent meta-analysis on the efficiency of acute normovolemic hemodilution (ANH) (1), this strategy is still often considered in the perioperative setting to reduce blood transfusion (1-4). Although there is no consensus in the literature about the threshold level for the lowered hematocrit (Hct), the critical level to initiate transfusion in pediatric and adult population differs (5-11) and is only based on expert opinion rather than evidence-based findings.

When achieving ANH, various fluid replacements have been considered in order to maintain hemodynamic stability. However, administration of crystalloids or colloids leads to fluid extravasation, thereby affecting various organs, with the heart and the lungs being the most affected due to cardiopulmonary interactions (12,13). We recently demonstrated that fluid resuscitations with crystalloids and colloids lead to perivascular pulmonary edema with subsequent adverse alterations in lung tissue mechanics (13).

The main factors in the development of pulmonary edema are related to capillary permeability, transmural hydrostatic pressure and the oncotic pressures determined by the protein balance. Different cellular profiles in the bronchoalveolar lavage fluid were observed between children and adults with normal (14) or diseased lungs (15), suggesting an age-related difference in the alveolar-capillary permeability following fluid replacement therapy. While this also implies potential dissimilarities between children and adults in the pulmonary edema development and subsequent impairment of lung function, the changes in the cardio-respiratory system to ANH between a pediatric and an adult population have not been compared. We hypothesize that the difference in the age-related physiological behavior will be reflected in the cardiorespiratory

changes following ANH. Therefore, we aimed at characterizing the age-related differences in the cardiorespiratory responses to ANH in a porcine model with particular focus on the separate description of the F mechanics and respiratory tissue viscoelasticity.

METHODS

Animal preparation

Following approval by the institutional ethics committee for experimental research of the University of Geneva and animal well-fare committee of the Canton of Geneva, Switzerland (registration number GE/44/14), studies were performed on 5-week old piglets (n=8) and 5-6 years old adult mini pigs (n=8). Considering that pigs age at a rate of about 5 years to one of a human, these ages reflect infants for the piglets (under 1 year of age) and maturity (30 years)(16). Since our primary aim was to compare the changes in the adult and pediatric model and not to characterize alterations in the respiratory mechanics or hemodynamics *per se*, no sham-treated control group was involved.

All animals were premedicated by an intramuscular injection of a mixture of azaperone (8 mg/kg), midazolam (0.75 mg/kg) and atropine (25 μg/kg). Induction of anesthesia is achieved with isoflurane 3-4% until securing intravenous access via the ear vein then, tracheal intubation was performed after administration of fentanyl (2 μg/kg) and atracurium (0.5 mg/kg). Anesthesia was maintained by continuous intravenous infusions of propofol (10-15 mg/kg/h) and fentanyl (10 μg/kg/h) via the ear vein. Animals were mechanically ventilated with volume controlled mode (7 ml/kg) by a commercial ventilator with additional software (Servo-i, Maquet Critical Care, Solna Sweden), with a frequency of 12-15/min in the adult group and 25-30/min in the piglet group in order to obtain an end-tidal CO_2 ($ETCO_2$) in the normal range. A positive end-expiratory pressure (PEEP) at 5 cmH_2O was kept in all animals and the inspired oxygen fraction (FiO_2) was set to 0.3. After ensuring adequate anesthesia and analgesia level, via surgical preparation the right femoral artery was cannulated with a thermistor-tipped femoral arterial catheter (Pulsiocath 5F, 20 cm or 3F, 7 cm) for continuous arterial blood pressure monitoring and hemodynamic measurements using PiCCO monitoring system (PiCCO Plus, Pulsion Medical Systems, Germany). The left femoral artery was also cannulated for

blood sampling and to perform blood withdrawals for hemodilution. In addition, the right internal jugular vein was cannulated with a triple-lumen central venous catheter (Arrow, USA) for fluid administration, cold fluid indicator injections and for central venous pressure monitoring. Rectal temperature was monitored with a temperature sensor (Thermalert, model TH-8, Physitemp, Clifton, NJ, USA) and was maintained at 38°C with a heating pad (Miostar, Zürich, Switzerland). Airway, arterial and central venous pressures, heart rate and electrocardiogram were continuously displayed and recorded via PowerLab data acquisition hardware (PowerLab, ADInstruments, Oxfordshire, UK), and recorded on a computer with LabChart software (ADinstrument, Dunedin, New Zealand).

Arterial blood was collected at control condition and after each step of hemodilution to determine pH, partial pressure of O_2 (PaO_2) and CO_2 ($PaCO_2$), bicarbonate (HCO_3) and base excess (BE) (VetScan i-STAT1 Handheld Analyzer with EG6+ cartridge, Abaxis, Union City, CA, USA). Bladder catheterization was performed to ensure free urinary outflow and to avoid possible urinary retention.

The core body temperature was maintained stable by warmed fluids, warming lights and a heating pad.

Hemodynamic monitoring

Cardiac output (CO) and volumetric variables such as extravascular lung water (EVLW) were measured with the single-indicator transpulmonary thermodilution technique (17-20). Briefly, the PiCCO values were obtained by injections of 10 ml (in piglets) or 20 ml (in adult pigs) boluses of cold (< 5°C) normal saline via the central venous line and temperature changes were measured with the thermistor tipped femoral artery catheter. Analyses of the thermodilution curve allow the calculation of the cardiac output (CO) and extravascular lung water (EVLW)(21,22). EVLW reflects all fluid that is outside of the pulmonary vasculature which includes interstitial and alveolar fluid. As it was demonstrated previously rapid changes in blood

pressure or intravascular volume affects the accuracy of the PiCCO measurements unless recalibrated(23). Therefore each set of PiCCO measurement was performed by recalibration by transpulmonary thermodilution. Pulmonary blood flow (PBF) was estimated from the capnodynamic monitoring as described below.

Measurement of ELV and PBF

ELV, the effective lung volume participating in gas exchange, and PBF were calculated using the differential Fick method (CO_2) as described earlier (24,25). Briefly, a specific breathing pattern is used to create variations in the end tidal carbon dioxide (E_tCO_2) of about 0.5-1.0 kPa, which allows estimation of ELV and PBF using the differential Fick equation(26). This specific pattern of breathing is achieved by an additional software of the Servo-i ventilator that creates five consecutive alterations in inspiratory/expiratory ratio (1:2 to 1.5:1) by varying the inspiratory pause (27). Airflow and expired CO_2 are measured by the ordinary Y-piece flow sensor and a main stream CO_2-transducer. Flow and CO_2 data from Servo-i are exported, via the RS232 port, to a laptop with a special designed software application written in Matlab™ (Mathworks, Natick, Massachusetts, U.S.A.).

Impedance measurements

The input impedance spectra of the respiratory system (Zrs) in the animals was measured using a method previously described (28,29). Briefly, the tracheal cannula was connected to a loudspeaker-in-box system at end-expiration, which was pressurized to the level of PEEP during the measurements to maintain the mean transpulmonary pressure constant during measurements. Small amplitude pseudorandom signal (15 non integer multiples between 0.5 and 21 Hz) was generated by a loudspeaker and was led through a screen pneumotachograph (11 mm ID) connected to a differential pressure transducer (model 33NA002D, ICSensors, Malpitas, CA, USA) to measure tracheal airflow (V’). Another pressure transducer connected

to a side-port of the endotracheal tube with identical type was used to measure the airway opening pressure (Pao). Zrs (Zrs = Pao/V') was calculated by Fast Fourier transformation with 4-s time windows and 95% overlapping from the 10-s long recordings.

To separate airway and respiratory tissue mechanics from Zrs spectra, a model containing frequency-independent airway resistance (Raw) and inertance (Iaw), in series with a constant-phase tissue model (30) including damping (G) and elastance (H) was fitted to Zrs by means of a global optimization procedure, which minimized the differences between the measured and modeled impedance values. As previously established, Raw reflects mainly the flow resistance of the airways, G describes the energy loss within the respiratory tissues (resistance) while H characterizes the energy storage capacity of the respiratory tissues (elastance). The reported Raw values were corrected for the resistance of the measurement set-up, including the tracheal cannula.

Study protocol

Following animal preparation and after reaching steady-state conditions in the systemic hemodynamic and ventilation parameters, two hyperinflation manoeuvres were performed by superimposing three inspiratory cycles to reach a peak pressure of 30 cmH_2O to standardize the volume history. Three minutes later, arterial blood samples were taken for blood gas analyses and a set of respiratory mechanical data including 4 Zrs recordings, and hemodynamic parameters were recorded to establish the baseline values (BL). Continuous measurement of ELV and PBF with the Servo-i ventilator was then performed for 10 minutes. In the next 20-min period, stepwise 10 ml/kg of arterial blood withdrawal manoeuvres were achieved while compensating with 30 ml/kg of crystalloids solution (Ringer acetate®) (Figure 1). Another lung volume standardization maneuver was performed 2 min before starting data collection. Arterial blood samples were taken and another set of respiratory mechanical and systemic

hemodynamic, ELV and PBF measurements were performed following achieving of at least 10 minutes of steady state condition. This sequence, which had a duration of 30 minutes in total, was repeated 7 times (ANH1-ANH7 in piglets) or until severe hemodynamic impairment characterized by a drop in MAP below 50 mmHg (5 times in adult pigs, as ANH1-ANH5).

Statistical analyses

Unless indicated differently, group mean values with SE data are reported. Normality was checked with the Kolmogorov-Smirnov test with Lilliefors correction. To assess the effect of hemodilution in the two groups of animals, two-way repeated measures analysis of variance (ANOVA) with a general linear model was used with group allocation as independent factor (piglet or pig), and the measurement condition (baseline and hemodilution manoeuvers) as repeated measures (within-subject) factor. The interaction between these factors was also incorporated in the analyses (i.e. group-by-hemodilution level). Since the changes in the parameter values relative to the baseline were in the center of interest, the repeated comparisons were performed between the parameters obtained under the baseline conditions (BL) to those measured following each step of hemodilution (ANH1-ANH7). The Holm-Sidak multiple comparison procedure was employed to compare parameters under different conditions. Correlation analyses were performed by using Pearson correlation test. A sample size estimation was performed considering 25% absolute difference between the groups as clinically significant, and taking into account the mean values and the variabilities of the primary outcome (H) established in our previous experiments in pigs (33.1±4.5 cmH_2O/l)(28) and the expected mean±SD values for piglets of 3 times higher. This sample size estimation based on a standardized effect size of 0.92 revealed that 8 pigs are necessary in each group to detect statistically significant difference with α to be 0.05 and 85% power with adjustments for multiple comparisons (31). The statistical tests were performed with a SigmaPlot (Version 11,

Systat Software, Inc. Chicago, IL, USA) software package. In each test, a significance level of $p<0.05$ was applied.

RESULTS

Baseline parameters including the body weight, systemic and pulmonary hemodynamics, and respiratory mechanics are shown in Table 1 for both study populations. As expected from the higher body weight, adult pigs exhibited higher CO, PBF and ELV levels, whereas respiratory mechanical parameters including Raw, G and H were lower.

Changes in those parameters where markedly different magnitudes are expected between the study populations are presented as relative changes to the baseline following each step of ANH; this facilitates direct comparisons between the trends obtained in adult pigs and piglets. The exact values for these parameters can be anticipated in the view of the baseline parameters presented on Table 1.

Changes in the respiratory mechanical parameters are shown in Figure 2. Statistically significant interactions were observed between age and hemodilution steps for all respiratory mechanical parameters ($p<0.05$, $p<0.01$ and $p<0.001$ for Raw, G and H, respectively), indicating that the age affects significantly the respiratory mechanical responses to ANH. Statistically significant differences between the study groups were observed following the ANH maneuvers in Raw ($p<0.01$ after the second ANH maneuver), G ($p<0.03$ after the first ANH maneuver), and H ($p<0.05$ after the third ANH maneuver). Concerning the changes in the parameters within a protocol group, ANH led to immediate increases in Raw ($p<0.001$) and H ($p<0.001$) in both study groups. Conversely, ANH led to immediate and significant decrease in G ($p<0.01$) in the adult pigs whereas, piglets exhibited less pronounced drops (Figure 2 B). Analyses of the interactions revealed that changes in ELV over ANH levels were not significantly different between groups ($p=0.7$), with significant within group decreases occurred only in the adult population (Figure 2 D, $p<0.05$).

Changes in the hemodynamical parameters obtained from the adult and pediatric porcine population are demonstrated on Figure 3. Progressive ANH decreased gradually the Hct level in both study groups ($p<0.02$), with no significant effect of age ($p=0.13$) (Figure 3 A). Compensating the blood extraction with crystalloids led to changes in EVLW in both groups that were more pronounced in the adult pigs (Figure 3 B), leading to significant differences between the groups after at the final stage of the protocol (group-by-hemodilution interaction of $p<0.01$). While HR increased ($p<0.001$ for ANH effect) similarly in both groups concomitant to the hemodilution ($p=0.94$ for group-by-hemodilution interaction), MAP remained stable in piglets as a result of significant increase in CO (Figure 3 E, $p<0.02$). Conversely, MAP exhibited significant decreases ($p<0.03$) in adult pigs following hemodilution as a result of drop in PBF (Figure 4 F, $p< 0.005$), which was not compensated by the increase in HR (Figure 3 D). Due to dissociated changes in MAP in the protocol groups, age had significant effects on the MAP responses to ANH ($p<0.001$ for group-by-hemodilution interaction).

Parameters obtained from the arterial blood samples are demonstrated on Figure 4. No statistically significant differences were detected between the study groups in pH, $PaCO_2$, HCO_3 and BE. However, PaO_2 exhibited statistically significant decreases following each ANH step only in the adult pigs ($p<0.02$). Lactate concentration was obtained at the end of the experimental protocol and revealed levels in the normal range for both adult pigs (1.63±0.71 mmol/l (n=6)) and piglets (0.63±0.25 mmol/l (n=7)).

DISCUSSION

The results of the present study revealed fundamental differences between the adult and pediatric porcine population in response to stepwise acute normovolemic hemodilution in respiratory mechanics, systemic and pulmonary hemodynamics, lung edema formation and loss of effective lung volume participating in gas exchange. Compared to piglets, ANH induced more pronounced changes in the airway and respiratory tissue mechanical parameters in the adult pigs. Stepwise hemodilution increased heart rate similarly in both groups with significant differences in the responses in CO and MAP. In adult pigs, ANH induced extravascular lung water accumulation that led to loss in lung volume and decreases in the arterial oxygen tension.

Effects on respiratory function

Respiratory mechanical parameters deteriorated in both age groups, with more severe alteration in the adult population. Since acute fluid overload elevates Raw and H (32), the initial increases in Raw and H observed in the present study can be attributed to the fluid overload as a result of the cardiopulmonary interaction. The rises in H with no further elevations in Raw after the 2nd hemodilution step may be attributed to the appearance of fluid extravasation. The magnitude and dynamics of the mechanical changes are in agreement with the observed increases in EVLW and the diminishment of ELV. These changes reflect pulmonary edema formation with lung volume loss affecting PaO_2 in pigs. The link between H and EVLW is confirmed by the significant correlation between the changes in these parameters following ANH ($r=0.44$, $p<0.05$).

Interestingly, G decreased significantly following hemodilution. This mechanical parameter is related to the resistive losses induced by the internal friction in the respiratory tissues. This finding may be related to decreased cell content of the blood in the pulmonary circulation (33).

The subsequent decreases in erythrocyte density may reduce cell-to-cell interaction thereby lessening energy dissipation in the blood-filled respiratory tissues. Accordingly, the decreases in Hct relative to the baseline correlated significantly with those in G in the initial phase of the protocol before fluid extravasation (i.e. before the 4^{th} hemodilution maneuver) ($r = 0.38$, $p<0.01$).

Effects on hemodynamics

A common finding in both groups of animals is the presence of a compensatory cardiovascular mechanism maintaining physiological systemic hemodynamics until a drop in hematocrit to a level of 17% (Figure 3). Accordingly, ANH increased HR and CO as a compensatory mechanism to the reduced hemoglobin and oxygen content of the blood. The efficiency of this compensation can be appreciated by the maintenance of homeostasis and lack of metabolic acidosis.

Differences between the age groups

The most remarkable finding of the present study is the blunted adverse cardiorespiratory responses in piglets despite the similar decreases in Hct. The greater extravasation of intrapulmonary fluid in the adult model explains this age-dependence. Furthermore, the relative proportion of the contribution of the invariable blood viscoelasticity to the overall tissue damping properties of the lungs is expected to be greater in adult pigs than in piglets. As a consequence, significantly greater drops in G were observed in pigs than in piglets.

Concerning the hemodynamic response to ANH, the two groups behaved differently beyond the threshold level of 17% in hematocrit. While the piglets were still able to compensate the further drop in hematocrit (as low as 11%), adult pigs exhibited severe hemodynamic deteriorations. This finding can be explained in the adult model by the development of cardiac failure due to the severe decrease in oxygen transport. Considering the increased myocardial

oxygen demand due to severe tachycardia and to the decrease in tissue perfusion due to the elevated vascular permeability, a decrease in hematocrit level is expected to jeopardize rapidly the cardiac function in adults. Since younger individuals tolerate better the increased oxygen demand of the myocardium (34), this adverse cardiopulmonary effect is expected to be less prominent in this age group.

Methodological considerations and limitations

A fixed blood volume (10 ml/kg) was removed in both study populations. It is of note that the blood volume expressed relative to body weight is about 30% greater in piglets than for adults (35,36). While this phenomenon may explain the initial steeper drops in the Hct in the adult pigs (Figure 3 A), the level of targeted Hct was comparable between the study populations, suggesting that the degree of hemodilution was comparable between the groups.

Replacement of blood with a crystalloid is commonly applied to preserve normovolemia following acute blood loss. However, there have been only scarce objective assessments regarding the optimal fluid replacement strategy contributing greatly to the generation of recent debates around this conflicting issue. Recent international consensus recommendations promote the administration of crystalloids as fluid replacement in the presence of acute blood loss (37). In agreement with these guidelines, we applied 3:1 ratio that prove to maintain a stable homeostasis with no metabolic acidosis (Figure 4) indicating maintenance of oxygen metabolism and sufficient systemic perfusion. Furthermore, efforts were made to maintain respiratory stability by regular volume standardization maneuvers, to minimize any potential time effect of anesthesia and mechanical ventilation *per se*. However, it cannot be excluded that some of the cardiorespiratory and hemodynamic changes observed over time in each group was related to the anesthesia itself; this potential further limitation could have been addressed by using an anesthetized control in each cohort.

Summary and conclusions

The results of the present study demonstrate prompt effects of ANH on the cardiorespiratory function with the severity of adverse symptoms depending greatly on the age of the subject. In adult pigs, gradual developments of bronchoconstriction, lung tissue extravasation, lung tissue stiffening, and deteriorations in systemic and pulmonary hemodynamics were observed, with rapid exacerbation of these adverse symptoms when hematocrit was lowered below a threshold limit of 17%. Conversely, these detrimental changes in the cardiorespiratory system were markedly milder in the pediatric porcine model. Therefore, the age-dependent cardiorespiratory responses to ANH suggest a better tolerability of this intervention in young individuals; however, a clinical study is needed to clarify the applicability of these findings to humans.

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	Piglets (n=8)	Adult pigs (n=8)	Absolute differences Mean (95% CI of the difference)
BW (kg)	9.4±0.85	54.3±7.3	44.9 (39.3 – 50.5)
MAP (mmHg)	65.5±14.4	79.0±13.9	13.5 (-1.7 – 28.7)
FCO (l/min)	1.7±0.40	2.8±0.82	1.1 (0.41 – 1.79)
PBF (l/min)	1.80±0.54	4.83±1.3	3.03 (1.96 – 4.10)
HR (1/min)	97.6±16.7	119±31	21.4 (-5.30 – 48.1)
ELV (ml)	275±62	1702±238	1427 (1240 – 1614)
ELV (ml/kg)	29.2±5.4	31.5±3.1	2.3 (-2.42 – 7.02)
EVLW (ml)	183±79	200±31	17 (-47.4 – 81.4)
Raw ($cmH_2O.s/l$)	3.19±0.51	1.32±0.14	1.87 (1.46 – 2.27)
G (cmH_2O/l)	23.9±3.4	6.08±1.5	17.82 (15.0 – 20.6)
H (cmH_2O/l)	111±14.7	31.3±5.9	79.7 (67.7 – 91.7)

Table 1. Baseline characteristics of the piglets and adult pigs. Data in the first two columns are mean ± SD. Last column reports the absolute value of the differences of means and the 95% confidence intervals (CI) for the differences. BW: body weight, MAP: mean arterial pressure, CO: cardiac output, PBF: pulmonary blood flow, HR: heart rate, ELV: effective lung volume, EVLW: extravascular lung water, Raw: airway resistance, G: tissue damping, H: tissue elastance.

FIGURE LEGENDS

Figure 1. Scheme of the experimental protocol. Control measurements were initiated after establishing stead-state respiratory and hemodynamical conditions. Measurements were repeated after each step of acute normovolemic hemodilution (ANH1-ANH7). FOT: forced oscillatory recordings to assess airway and tissue mechanics, ELV: effective lung volume, PBF: pulmonary blood flow, CO: cardiac output, EVLW: extravascular lung water, MAP: mean arterial pressure, HR: heart rate.

Figure 2. Changes in the airway resistance (Raw, panel A), tissue damping (G, panel B), tissue elastance (H, panel C) and effective lung volume (ELV, panel D) following stepwise acute normovolemic hemodilution (ANH1-ANH7) in adult pigs (n=8; open symbols) and in 5-week old weaning piglets (n=8; closed symbols). Data points and error bars represent mean±SE values. *: $p<0.05$ vs. the corresponding baseline values. †: $p<0.05$ between groups within a measurement condition.

Figure 3. Hematocrit levels (Hct, panel A) and relative changes in the extravascular lung water (EVLW, panel B), mean arterial pressure (MAP, panel C), heart rate (HR, panel D), cardiac output (CO, panel E) and pulmonary blood flow (PBF, panel F) following stepwise acute normovolemic hemodilution (ANH1-ANH7) in adult pigs (n=8; open symbols) and in 5-week old weaning piglets (n=8; closed symbols). Data points and error bars represent mean±SE values. *: $p<0.05$ vs. the corresponding baseline values. †: $p<0.05$ between groups within a measurement condition.

Figure 4. Parameter values obtained from the arterial blood samples following stepwise acute normovolemic hemodilution (ANH1-ANH7) in adult pigs (n=8; open symbols) and in 5-week

old weaning piglets (n=8, closed symbols). PaO_2: partial pressure of oxygen, $PaCO_2$: partial pressure of carbon dioxide, HCO_3: bicarbonate, BE: base excess. Data points and error bars represent mean±SE values. *: $p<0.05$ vs. the corresponding baseline values. †: $p<0.05$ between groups within a measurement condition.

Figure 1

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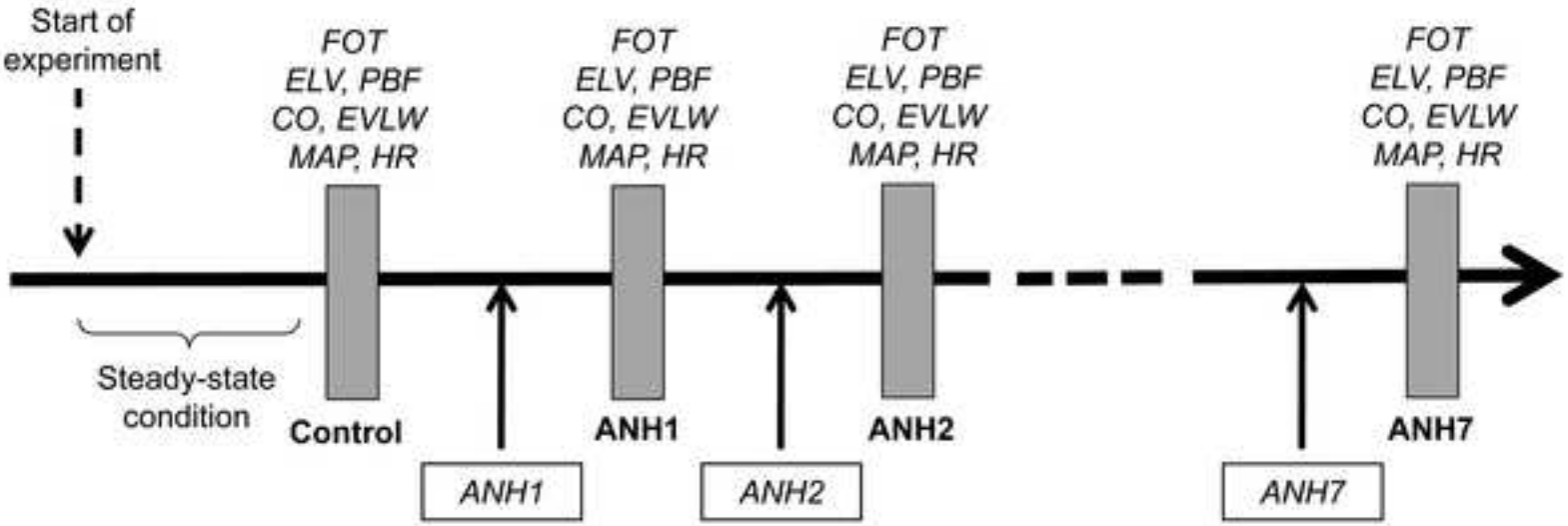

Figure 1

Figure 2

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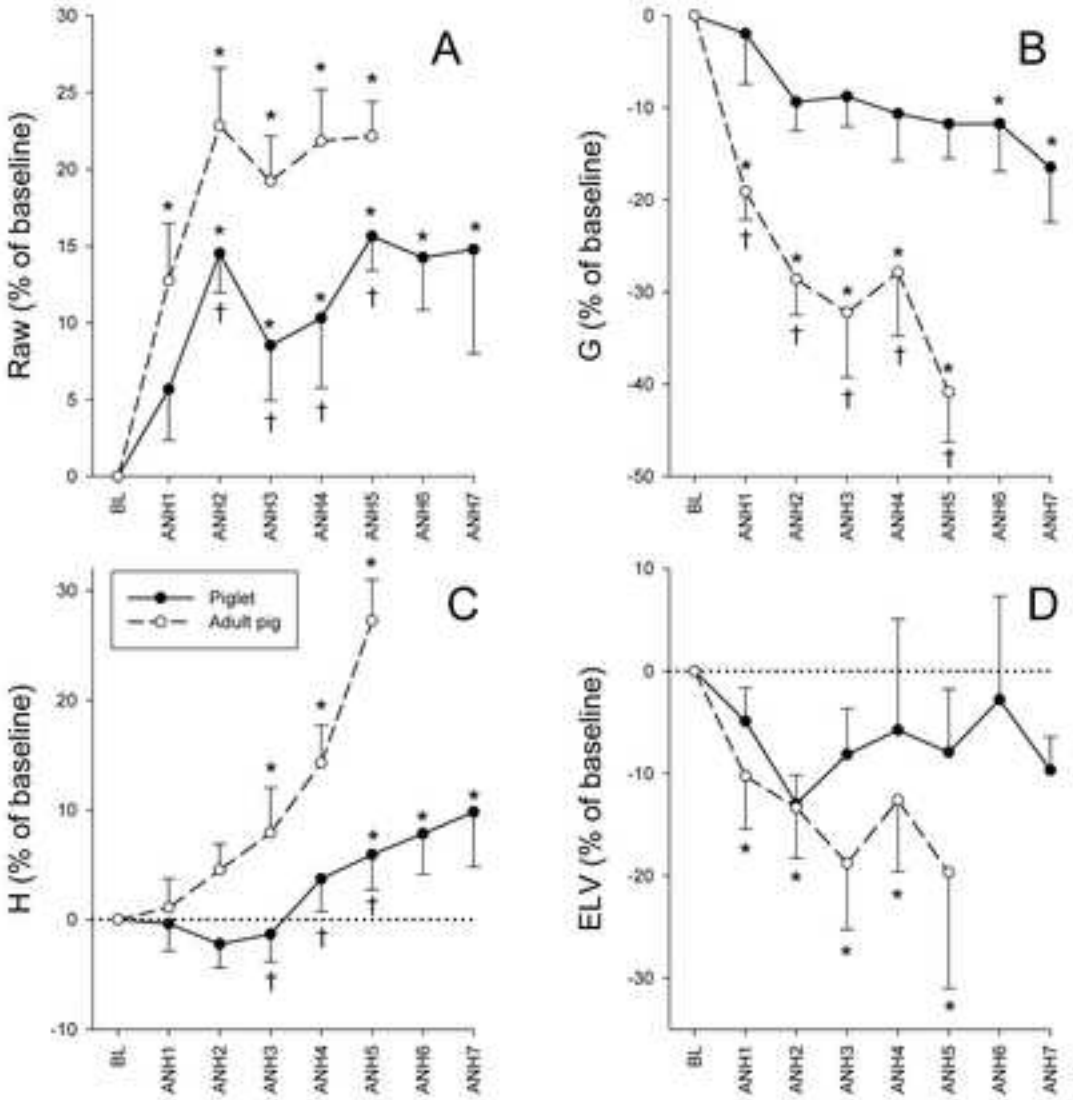

Figure 2

Figure 3

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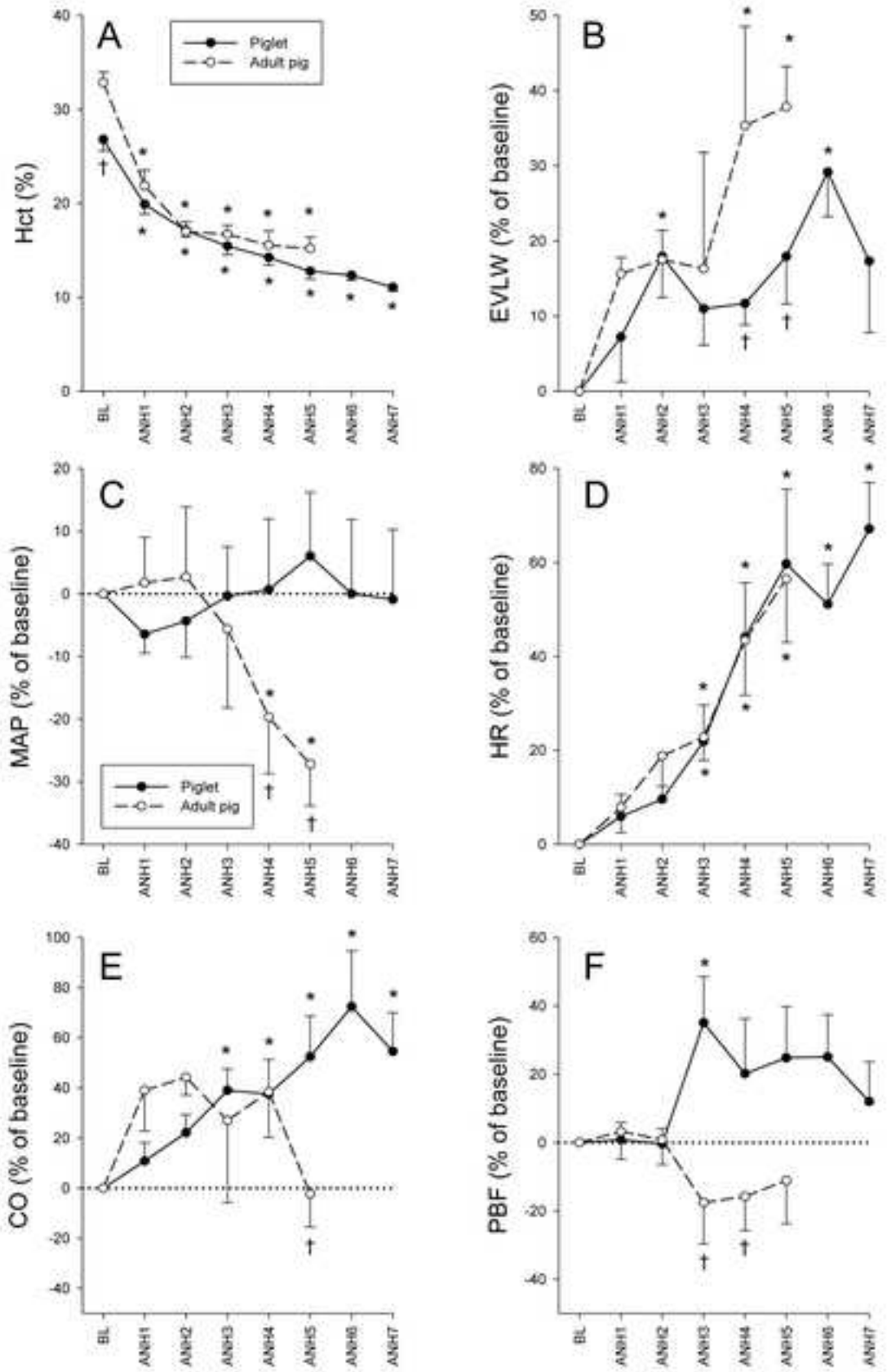

Figure 3

Figure 4

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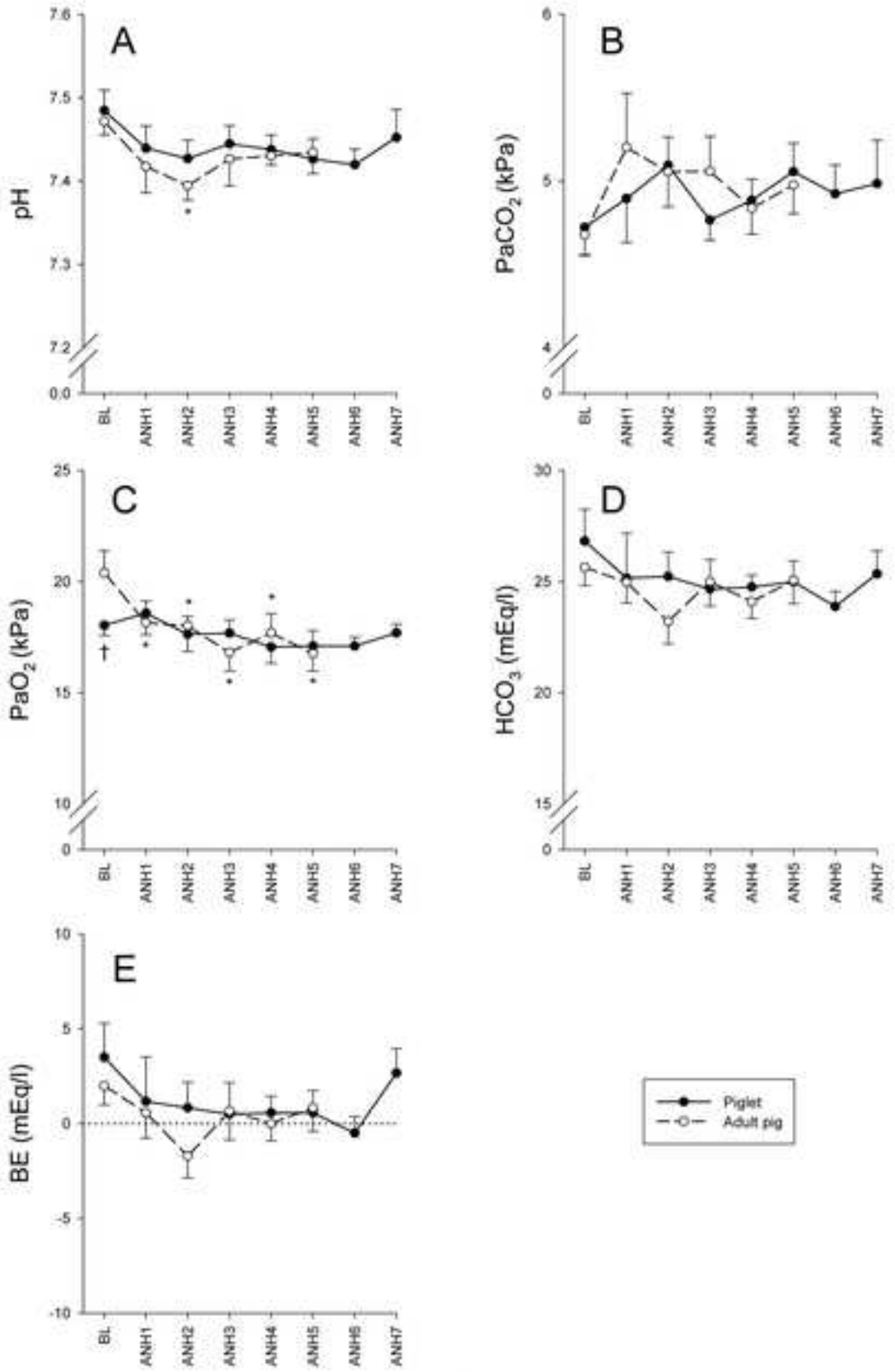

Figure 4

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

Carol Kilkenny[1], William J Browne[2], Innes C Cuthill[3], Michael Emerson[4] and Douglas G Altman[5]

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	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Cover page
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Page 1
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	Pages 2-3
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Page 2
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Page 4
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	Page 4
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	Pages 4-8
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Page 4

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	Page 4
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	Page 4
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	Page 4
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Pages 5-7
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	Page 8
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Page 9
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	Page 9-10
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Pages 9-10
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	N/A
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	Pages 11-14
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Page 15
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Cover page

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
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